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(54) Title: NOVEL APPLICATIONS OF NICKEL NITRILOTRIACETIC ACID (NI-NTA) RESIN: HEMEPROTEIN REMOVAL, RECOVERY, AND PURIFICATION FROM BIOLOGICAL SAMPLES

(57) Abstract: The present invention relates to a method for the removal, recovery, and/or purification of hemeproteins or heme-protein-hemeprotein ligand complexes from biological samples using Ni-NTA resin (nickel nitrilotriacetic acid). Furthermore, the present invention pertains to a method for removing hemoglobin from his-tagged fusion proteins produced in a rabbit reticulate lysate. In addition, the present invention relates to a method for the preparation of a stromal free blood substitute and methods for the removal of extracellular hemeproteins or hemeprotein-hemeprotein ligand complexes. The present invention further relates to a solid support, comprising materials and reagents described as above.

Novel applications of nickel nitrilotriacetic acid (Ni-NTA) resin: Hemeprotein removal, recovery, and purification from biological samples.

The present invention relates to a method for the removal, recovery, and/or purification of hemeproteins or hemeprotein-hemeprotein ligand complexes from biological samples using Ni-NTA resin (nickel nitrilotriacetic acid). Furthermore, the present invention pertains to a method for removing hemoglobin from his-tagged fusion proteins produced in a rabbit reticulate lysate. In addition, the present invention relates to a method for the preparation of a stromal free blood substitute and methods for the removal of extracellular hemeproteins or hemeprotein-hemeprotein ligand complexes. The present invention further relates to a solid support, comprising materials and reagents described as above.

Several documents are cited throughout the text of this specification. The disclosure content of each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) is incorporated herein by reference.

The earliest reported use of immobilized metal affinity chromatography (IMAC) procedure involved low molecular weight organic compounds as chelators, covalent bound onto chromatographic supports to capture metal ions in industrial effluents (Meinhardt, 1949; Helfferich, 1961). Everson et al. (1974) discovered that metal ions coordinated to immobilized chelators can have application for the isolation of metalloproteins. Several subsequent works (Porath et al., 1975; Porath et al., 1983; Anderson et al., 1987; Winzerling et al., 1996) extended the practical application of this technique for the separation of serum proteins with the introduction of immobilized iminodiacetic acid (IDA) and several related tridentate ligand systems. The IMAC procedure relies on the ability of immobilized metal ion chelate complexes to interact with the side chain moieties (thio, carboxyl, imidazole) of specific amino acids accessible at the surface of proteins (Hutchens

et al., 1987; Berna et al., 1998). The coordinative interactions have been described as lone pair Lewis acid-Lewis base donor-acceptor interactions and typically involve the use of tridentate or tetradentate ligands such as IDA, nitrilotriacetic acid (NTA), tris-(carboxa-methyl)-etylenediamine (TED), O-phosphoserine (OPS), or carboxymethyl aspartic acid (CMA) with so-called "soft" or "borderline" metal ions such as Cu^{2+}, Zn^{2+} or Ni^{2+} (Zachariou and Hearn, 1996; Jiang et al., 1998). The binding behavior of various proteins has also been investigated by charging various chelate resins with nitrate salts of manganese, cobalt and chromium (Jiang et al., 1998).

Immobilized metal ion affinity chromatography (IMAC) has been explored as a probe into the topography of histidyl residues of a protein molecule. An evaluation of the chromatographic behavior of selected model protein-thioredoxin, ubiquitin, calmodulin, lysozyme, cytochrome c, and myoglobin on immobilized transition metal ions (Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) allows establishment of the following facets of the histidyl side chain distribution: (i) either interior or surface; (ii) when localized on the surface, accessible or inaccessible for coordination; (iii) single or multiple; (iv) when multiple, either distant or vicinal. Moreover, proteins displaying single histidyl side chains on their surfaces may, in some instances, be resolved by IMAC; apparently, the microenvironments of histidyl residues are sufficiently diverse to result in different affinities for the immobilized metal ions. IMAC, previously introduced as an approach to the fractionation of proteins, has become also, upon closer examination, a facile probe into the topography of histidyl residues. This is possible because of the inherent versatility of IMAC; an appropriate metal ion (M^{2+}) can be selected to suit the analytical purpose and a particular chromatographic protocol can be applied (isocratic pH, falling pH, and imidazole elution).

In recent years IMAC has enjoyed routine and widespread use in purification of proteins and peptides that have hexa or penta histidine residues appended to their amino or carboxy terminus by recombinant DNA techniques (his-tagged proteins) which invoke elution in buffers comprising 0.2-1M imidazole (Qia Expressionist Handbook, 1999). In the present invention, immobilization of hemeprotein does not

require modification of the protein sequence. In contrast to isolation of human serum albumin and transferrins which are achieved by binding with loading buffers at physiological ionic strength and pH and elution profiles at high ionic strength and either high or low pH (Anderson et al., 1987; Winzerling et al., 1996; Zachariou and Hearn, 1996) the hemeprotein separations described herein are primarily dependent on imidazole sensitive interactions between the native hemeprotein and immobilized nickel chelate over a broad range of pH and ionic strength. This feature enables immobilization of hemeprotein with greater selectivity than serum proteins because specific histidine residues in the vicinity of the heme prosthetic group are implicated in the coordination with metal ion rather than ionic and hydrophobic effects at the protein surface. The precise chemical interactions and coordination bonds between metal ion sphere and the hemeprotein have not been defined in the present work. It can be deduced from resolved crystal structures of myoglobin (Smerdon et al., 1990; Krzywada et al., 1998) and crystals of human hemoglobin (Fermi et al., 1984; Bruno et al., 2000) that the two histidine residues in proximity to each heme pocket, 3-4Å His-Fe distance, 6.4 Å His-His, for a total of 4 Fe- 8 histidines per tetramer residues are not involved in the stabilization of bound oxygen and may be available to participate as donor ligands with the chelate in hexadentate coordination of nickel metal ion. Histidine side chains accessible on the surface of the protein show greater inter-residue distances and would not necessarily account for the observed stringency of hemoglobin and myoglobin binding to Ni-NTA in buffers containing equal to or lower than 20mM imidazole.

There are numerous reports on immobilized metal ion affinity chromatography (IMAC) of human serum albumin, transferrins and non-hemeproteins, yet there are very few studies of hemeproteins in IMAC. Two publications involve application of immobilized metal ion chelate complexes for adsorption and chromatographic separations of human serum proteins and hemeproteins myoglobin and cytochrome c (Zacharaiou et al., 1995; Jiang et al., 1998).

The IMAC behavior of horse heart myoglobin, tuna heart cytochrome c and hen egg lysozyme was investigated with immobilized hard Lewis metal ions Al^{3+},

Ca^{2+}, Cu^{2+}, Fe^{3+} and Yb^{3+} on O-phosphoserine (POS), 8-hydroxyquinoline (8HQ) and iminodiacetic acid (IDA) coupled to sepharose CL-4B. Adsorption of cytochrome c to the chelates was done in equilibration loading buffer; 30mM HEPES 3mM imidazole at pH 5.5 and pH 8 whereas myoglobin binding occurred only in buffer at low pH due to its different surface charge characteristic. IMAC of both hemeproteins showed pseudocation exchange at slightly higher than physiological ionic strength. Binding and elution was achieved from the abovementioned immobilized chelates without metal ion and the introduction of various metal ions (i.e. Mn^{2+}) gave only modest improvement in binding selectivity over a very narrow range of salt concentration, 0.1-0.3M NaCl.

In the second publication, Jiang et al. (*loc. cit.*) attempt to improve chromatographic selectivity of a immobilized metal chelate system for horse heart cytochrome c, horse defibrinated human plasma. They introduce a novel metal ion chelating ligand 1,4,7-triazacyclononane (tacn). The properties of this metal chelate complexed to metal ions Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Cr^{3+} are documented and the influence of pH and ionic strength on the adsorption to immobilized tacn were investigated. Data from batch adsorption experiments are presented as percentage of bound to total protein (B/T) versus pH, range 4 to 10 and B/T versus ionic strength, range 0 to 3M NaCl. Adsorption of myoglobin at pH 4 to 7 in 0.5M NaCl was found on tacn free of metal ion at 20 to 40% and on tacn –Mn^{2+} or tacn –Cr^{3+} the binding increased to 80%. No myoglobin binding occurred at pH ≥8.0 to tacn alone and some adsorption of myoglobin at pH 7 to 8 to immobilized Cu^{2+}tacn, Cr^{3+} tacn and $Ni2^{+}$ tacn was detected. In this system, binding preference of cytochrome c and myoglobin to Ni^{2+}tacn is greater than that of tacn chelate alone. However, the elution of hemeproteins was only demonstrated using high ionic strength buffer of 1 to 3 M NaCl and the maximum recovery from the adsorbent was 50% of the protein bound.

The principle feature of the immobilized metal ion tacn compared to other chelate systems is its larger stability for borderline metal ions and behavior as triprotic base which allows deprotonation at pH 2, pH 5.4 and pH 10. The latter property ensures that the chelate will not dissociate when it is complexed to metal ion

center except at very low pH. The system clearly lacks requisite selectivity for hemeprotein separations and as presented offers no advantage for either removal or purification of hemeprotein from biological samples.

In view of these deficiencies of the art, the technical problem underlying the present invention was to provide a rapid, robust, simple, and optionally pharmaceutically applicable method to specifically remove hemeproteins, preferably hemoglobin or myoglobin from biological samples preferably blood or blood substitutes. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a method of removing hemeproteins or hemeprotein-hemeprotein ligand complexes from a biological sample comprising (a) contacting said biological sample containing said hemeproteins or hemeprotein-hemeprotein ligand complexes with a Ni-NTA resin; (b) eluting components other than hemeproteins or hemeprotein-hemeprotein ligand complexes from said biological sample contacted with said Ni-NTA resin with a buffer containing 0 to 20mM imidazole and/or 0 to 3 M NaCl; and optionally, (c) eluting hemeproteins or hemeprotein-hemeprotein ligand complexes from said Ni-NTA resin with a buffer comprising imidazole in a concentration higher than 20mM; or with a buffer comprising nitric oxide in a concentration of about 0.1 to 1mM.

The term "hemeproteins" as used in accordance with the present invention denotes any protein carrying a heme group as a prosthetic group and which exists in an oxygenated as well as an deoxygenated state.

The term "hemeprotein ligand" refers to a group of compounds comprising endotoxins, haptoglobins, gases that bind to hemeproteins such as NO, sugars found in glycated hemoglobin and small molecules binding to hemeproteins.

The term "hemeprotein-hemeprotein ligand complex" refers to non-covalent interactions between said hemeprotein and said hemeprotein ligand that result in a binding and thus a complex formation.

The term "biological sample" as used herein comprises solid or liquid materials of plant or animal origin. Solid materials may be dissolved in physiological saline solution or appropriate buffers as, for example, Tris-buffer or phosphate buffers. Preferably the hemeprotein is myoglobin or hemoglobin. As is immediately evident to the person skilled in the art the methods and uses of the present invention are not only applicable to biological samples containing hemoglobin as described in the examples but may be easily adapted to other hemeproteins or hemeprotein-hemeprotein ligand complexes using known standard procedures of optimization (Hemdan et al., 1989).

The term "buffer" is intended to encompass all buffers suitable for the application of the different embodiments of the invention. The term "buffer" as used herein preferably denotes buffers comprising imidazole and/or NaCl but may be also devoid of imidazole and/or NaCl. Preferably, at least 5, more preferably, at least 10 mM NaCl are comprised in said buffer. In another preferred embodiment the buffer is a Tris-buffer or a phosphate buffer. Also preferred are buffers having a pH of about 5 to about 8. More preferably, the Tris-buffer comprises approximately 50mM Tris-HCl and 130 to 300mM NaCl and has a pH of about 8.0 and/or the phosphate buffer comprises approximately 50mM NaH_2PO_4, 50mM NaCl and has a pH of about 7.4.

Hemeproteins are present in blood and major organs to perform oxygen transport (i.e. hemoglobins) and are essential enzymes for mitochondrial respiration (e.g. cytochrome c), xenobiotic and hormone metabolism (e.g. cytochrome P450s). The release of hemeproteins outside of their native membrane or intracellular compartment can be toxic or indicator of severe and life threatening conditions. Hemoglobin operates at near saturation concentration inside red blood cells and individuals of high hematocrit experience hemolysis, usually well tolerated as a transient condition. However, chronic hemolysis and sustained elevation of plasma hemoglobin levels leads to release of heme - the major iron source responsible for iron-mediated oxidative damage in patients suffering genetic diseases of hemogblobin, iron transport proteins and enzymes of heme biosynthetic and degradative pathways. The high affinity of hemoglobin for bacterial derived

endotoxins pose a serious risk for sepsis and the capacity of plasma hemoglobin to bind and transport nitric oxide can lead to hazardous circulatory inbalances. The present invention demonstrates selectivity and high binding capacity of Ni-NTA to hemoglobin in human plasma and thus has potential implications for development of extracorporeal hemoglobin removal system in the clinic.

Fast, simple, easy and efficient means of hemeprotein binding and elution are described herein. The high binding capacity of Ni-NTA resin to hemoglobin in human plasma has implication for hemoglobin removal in the clinic and thus could provide solution to the problem of hemoglobin interference that is routinely encounted in measurements of clinical chemistry analytes either on Roche-Hitachi Systems or in offline tests of antigen antibody binding and enzymatic reactions that involve spectral endpoints. Furthermore, use of the method of the present invention for large scale hemoglobin purification can facilitate the production of stromal free blood substitutes and hemoglobin ligands such as haptoglobins.

Prior art production of haptoglobins involves multiple steps: HPLC purification of hemoglobin, immobilization of hemoglobin on cyanogen bromide activated sepharose and subsequent elution of the haptoglobins by protein denaturants such as guanidine hydrochloride. Immobilization of hemoglobin as described in this patent application will provide a selective and time-saving one-step capture of hemoglobin and haptoglobin hemoglobin complexes from whole blood lysate. The elution of haptoglobins can then be carried out without dissociation of hemoglobin from the Ni-NTA chelate, and thus yield haptoglobins of improved purity. This innovative methodology for production of haptoglobins of improved purity and at substantially reduced cost can have significant impact on the market for diagnostics of intravascular hemolysis (Dade-Boehring Clinical Chemistry reagent) and anti-haptoglobin antibody typing.

The incorporation of metal chelates such as Ni-NTA into solid supports or chips will lead to improved miniaturized electronic devices for specific detection of hemeprotein-hemeprotein ligand complexes such as glycated hemoglobin (HbAc1) or nitric oxide (NO) without compromising the chemical and physical properties

required for fluid sampling of blood and signal processing. Such devices in the clinic or home use have application for routine monitoring of blood sugar in patients suffering diabetes and renal and vascular diseases.

Demonstration of myoglobin binding to Ni-NTA is significant because currently there is a need for bedside plasma myoglobin detection in patients suffering myocardial injury and ischemia and to broaden the diagnostic value of this hemeprotein relative to other, less reliable plasma biomarkers.

The binding of hemoglobins to Ni-NTA resin shows selective elution profile with properties that allow improved hemoglobin purification compared to prior art methods of carboxymethyl cellulose ionic exchange chromatography, precipitation with organic salts and chemical and heat treatments.

The steps described in the method of molecular imprinting of hemeprotein or hemeprotein-hemeprotein ligand complexes on metal chelate solid support generates a unique template for hemeprotein or hemeprotein-hemeprotein ligand complexes. Recognition of protein structural and conformational states is achieved while specificity and stability of Ni-NTA interactions with the heme group and exposed histidines are retained.

Hemoglobin immobilized on Ni-NTA resin by direct contact between liberated hemoglobin and the resin, provides a first pass protein purification of a hemeprotein from a cell lysate or tissue sample. The present invention eliminates the need for differential centrifugation, ultra filtration, size exclusion chromatography and dialysis and offers commercial advantages of time saving and lower production costs. Further deficiencies of the prior art are the insufficient purity of hemoglobin preparations and contamination with endotoxin, bacteria or bacterial fragments or pyrogen. Prior art methods do not enable the synthesis of stable hemoglobin tetramers on immobilized adsorbent. The isolation of hemoglobin on Ni-NTA resin will help to minimize bacterial and pyrogen

contamination and to introduce conditions described in Roth et al. (1994) that are known to reduce endotoxin binding.

Here it has surprisingly been found that Ni-NTA binds hemeproteins present in different biological samples with high efficiency and specificity, providing the new methods and uses given in the embodiments. The method of the present invention will enjoy immediate practical laboratory value in hemeprotein purification and routine separation of proteins of desired biological function from hemeproteins like e.g. hemoglobin present in tissue samples, or heterogeneous reaction mixtures.

Existing hemoglobin preparations are often of insufficient purity and contain endotoxin, bacterial fragments or pyrogen. The isolation of hemoglobins on Ni-NTA resin will help to minimize the opportunity for bacterial and pyrogen contaminants and to introduce conditions described in Roth et al (1994) that are known to reduce the binding of endotoxin to hemoglobin.

In a preferred embodiment the biological sample is blood. In another preferred embodiment the animal is a mammal, preferably a human.

In a further embodiment the present invention relates to a method of removing hemeproteins from a his-tagged fusion protein produced in a rabbit reticulate lysate in vitro transcription-translation system comprising (a) contacting crude reticulate lysate containing said his-tagged fusion protein with a Ni-NTA resin; (b) eluting hemeproteins present in said crude reticulate lysate with a buffer comprising 20 to 80mM imidazole; and (c) eluting said his-tagged fusion protein with a buffer comprising 250mM to 1M imidazole.

The term "his-tagged fusion protein" as used herein is well known in the art. The application of his-tagged fusion proteins is, for example, described in the Qiagen Expressionist Handbook for high-level expression and purification of 6Xhis-tagged proteins. Third Edition (1999), Qiagen GmbH, Hilden.

The term "rabbit reticulate lysate in vitro translation transcription system" as used in accordance with the present invention is to be understood as for example used in "TnT®Quick coupled transcription/translation systems", Promega Corporation, Technical Manual No. 045, 1999, revised edition 9/99 and denotes in vitro coupled transcription/translation system that allow investigators to test cDNAs and designed gene constructs for their capacity to encode and produce properly folded protein molecules.

The results described in Example 3 demonstrate that Ni-NTA agarose has unique and dynamic binding properties; mainly that it 1) binds to hemeprotein in the presence of 10-20mM imidazole, and 2) permits the elution of said hemeprotein over a range of 20-80mm imidazole while retaining interactions between the hexa his sequence and Nickel nitrilotriacetic acid chelate. Because proteins differ in their requirements for folding and optimum expression the reticulocyte lysate system is often chosen as a convenient and quick way to test cDNAs and designed gene constructs for their capacity to encode and produce properly folded protein molecules. The Ni-NTA resin will thus allow investigators to examine radiolabeled his tagged proteins and peptides free of hemeproteins and reticulocyte lysate materials. This feature has advantages to examine 1) protein-protein interactions by immunoprecipitation experiments and 2) functional assays in which either the binding or biological activity of sub-microgram amounts of expressed protein might be perturbed by hemoglobin presence.

Prior art use of metal chelate resins like the proprietary NiNTA resin (Qiage GmbH, Hilden, Germany) or Zn-IDA has been exclusively for purification of 6X-his tagged proteins in biological preparations derived from bacterial, insect and mammalian protein expression systems (QIAexpression).

In a preferred embodiment at least one washing step is included after step (a) and prior to step (b). Washing solutions can be adapted according to the knowledge of the art.

In another embodiment the invention relates to a method of selectively enriching and/or purifying hemeproteins or hemeprotein-hemeprotein ligand complexes from a biological sample comprising contacting said biological sample containing said hemeproteins or hemeprotein-hemeprotein ligand complexes with a Ni-NTA resin; washing said biological sample contacted with said Ni-NTA resin with a buffer comprising imidazole in a concentration equal to or lower than 20mM; eluting hemeproteins from said Ni-NTA resin with (ca) a buffer comprising imidazole in a concentration greater than 20mM; or (cb) with a buffer comprising 0.1 to 1mM saturated nitric oxide; or (cc) with a buffer comprising reagents necessary to dissociate hemeprotein-hemeprotein ligand binding interactions, and optionally lyophilizing the eluate or crystallizing the hemeproteins or hemeprotein-hemeprotein ligand complexes from the eluate.

The term "purifying" as used herein refers to an enrichment in hemeprotein content to a content of at least 95%.

In a preferred embodiment elution is performed in step (ca) with a buffer comprising imidazole in a concentration of 25 to 80mM.

In another preferred embodiment at least two washing steps are carried out.

In a further preferred embodiment said washing steps are effected with a phosphate buffer or a Tris-buffer.

In another preferred embodiment the method comprises the further step of lysing whole cells in the biological sample prior to step (a).

In another embodiment the invention relates to a method for the removal of extracellular hemoglobin or hemeprotein-hemeprotein ligand complexes from patients affected with thalassemia, hemochromatosis, hemolytic anemia, or any other disease or condition in which the hemoglobin or hemeprotein-hemeprotein ligand complexes presence leads to injurious effects or patients suffering from hemoglobin-lipopolysaccharide septic shock, hemoglobin-nitric oxide mediated

septic shock, hemoglobin-nitric oxide induced imbalances, hemolysis conditions emanating from extra corporal oxygenation or prosthetic devices or exposure to drugs, chemicals, poisons or bacterial toxins comprising conducting peripheral blood from said patient through an extracorporeal hemoglobin or hemeprotein-hemeprotein ligand complexes adsorbent system, wherein a Ni-NTA resin adsorbent is built into an appropriate membrane tubing or sheet material to permit blood flow without lysis of red blood cells.

It is known to the person skilled in the art how to perform suitable tests which ensure clinical safety of the method of the present invention and how to meet concerns of possible nickel and nitrilotriacetic acid leakage into biological material as is for example described in Goyer et al. (1981) or Environmental Health Information Service US Department of Health on Human Services (2000). In this regard, it is important to note that Ni-NTA resins do not lyse or immobilize red blood cells, nor are they toxic nor do they induce coaggulation.

In a further embodiment the present invention relates to a method for the detection of hemeproteins or hemeprotein-hemeprotein ligand complexes in a sample, the method comprising (a) applying the sample to Ni-NTA immobilized on a substrate; (b) washing the immobilized Ni-NTA with a buffer comprising 0 to 20mM imidazole thereby eluting components other than hemeproteins or hemeprotein-hemeprotein ligand complexes from the immobilized Ni-NTA; (c) eluting hemeproteins or hemeprotein-hemeprotein ligand complexes from the substrate with a buffer comprising imidazole in a concentration higher than 20mM or with a buffer comprising nitric oxide in a concentration of about 0.1 to 1mM; (d) quantifying the eluted hemeproteins or hemeprotein-hemeprotein ligand complexes.

In a preferred embodiment said biological sample is whole blood lysate, plasma, serum, a tissue extract, a recombinant hemoglobin preparation, a blood substitute or any other sample containing hemeproteins.

The term "whole blood lysate" as used herein denotes a crude lysate of human or animal blood. For example, a whole blood lysate may be prepared by treatment of

whole blood with the detergent Saponin, optionally in the presence of sodium citrate in a concentration of about 0.5 to 1% (w/v).

The term "recombinant hemoglobin" as used herein is well known in the art. The preparation and properties of recombinant hemoglobin is inter alia described in Doyle et al., 1999.

The term "blood substitute" as used in the present description is also well known in the art. The nature and preparation of blood substitutes is inter alia described in Alayash, 1999 and Chang, 1997.

In a further preferred embodiment said patient is a mammal.

In a more preferred embodiment said mammal is human.

In another preferred embodiment said buffer in step (b) is a physiological buffer having a pH value of 5 to 8.

In a more preferred embodiment said pH value is between 7 and 8.

In another preferred embodiment said buffer in step (b) comprises 0.1 to 3M NaCl.

In yet another preferred embodiment the Ni-NTA resin is Ni-NTA agarose.

In another preferred embodiment said his-tag is a 6X-his-tag.

In still another embodiment the present invention relates to the use of a Ni-NTA resin for the removal of hemeproteins from a biological sample from a his-tagged fusion protein produced in a rabbit reticulate lysate in vitro transcription-translation system.

In a further embodiment the invention relates to the use of a Ni-NTA resin for the enrichment of hemeproteins or hemeprotein-hemeprotein ligand complexes from a biological sample.

In another preferred embodiment said biological sample is blood.

In a more preferred embodiment said Ni-NTA resin is Ni-NTA agarose.

In another embodiment the present invention relates to a method for the preparation of a stromal free blood substitute comprising the steps of (a) preparing a whole blood lysate; (b) contacting said whole blood lysate with a Ni-NTA resin; (c) eluting components other than hemoglobin from said biological sample contacted with said Ni-NTA resin with a buffer comprising imidazole in a concentration equal to or lower than 20mM; (d) cross-linking the resin-bound hemoglobin with an appropriate reagent in an appropriate buffer; (e) chemical modification of the resin-bound hemoglobin; (f) eluting the resin-bound hemoglobin from said Ni-NTA resin with a buffer comprising imidazole in a concentration equal to or higher than 20mM; and (g) gel-filtration in a size exclusion matrix.

In a preferred embodiment the elution in step (f) is performed with a buffer comprising imidazole in a concentration of about 20 to 200mM thereby separating hemoglobin tetramers from oligomers and uncross-linked monomers.

The term "gel-filtration" as used herein refers to methods well known in the art. Such methods are exemplified in Amersham Pharmacia Biotech Product Catalogue Chromatography Columns and Media (2000), Section 12, polypeptide 524-541.

The present invention provides methodology suitable for improving the preparation of blood substitutes. Direct contact between liberated hemoglobin and the resin provides a rapid first pass protein purification from a cell lysate or tissue sample and eliminates the need for differential centrifugation, ultra filtration, size exclusion

chromatography and dialysis, thus offering potential commercial advantages of time saving and lower production costs in large scale purification.

The method of the invention permits rapid isolation of stromal free native tetrameric hemoglobin on Ni-NTA resin or other support materials coated with Ni-NTA. The protein is thereby rendered a direct substrate in a stationary phase that is accessible to chemical reactions. Crosslinking reagents that introduce covalent bridges between alpha and beta chains resulting in the synthesis of stable tetramers are well known in the art. One of which is glutaraldehyde preferentially reactive with lysine residues of human hemoglobin. The application of glutaraldehyde is described in the examples below. Other chemical covalent modifications known in the art optimize the protein solubility, increase the efficiency of oxygen binding and introduce properties that are favorable for the production of blood substitutes (Manning, 1994; Alayash, 1999).

Because immobilization of hemeprotein is performed without detergents and under physiological conditions no loss of protein function or enzymatic activity is expected. In view of the diverse enzymatic activities known for hemeproteins as, for example, peroxidase, monooxygenase, cyclooxygenase, hydroxylase and dealkylase activity. The method of the present invention will be advantageously applied for performing catalysis with hemeproteins or appropriate hemeprotein derivatives immobilized on a surface (Hsu et al., 1999; Segal et al., 1999; Liang et al., 2000).

The present invention demonstrates that human hemoglobin may be directly chemically modified once immobilized on Ni-NTA agarose column. The immobilized human hemoglobin is treated with the cross-linking reagent glutaraldehyde and subsequently recovered after washings of the column and elution with buffer containing imidazole. Prior art methods for production of cross-linked stromal free hemoglobin are dependent on reactions done in solution. The advantage of carrying out modifications of hemoglobin on immobilized adsorbents is immediate isolation of the modified hemoglobin from the reactants. In the example given, cross-linked hemoglobin is purified from unreacted glutaraldehyde

and the monomeric, dimeric and oligomeric species are then eluted in imidazole and separated by size exclusion matrix in a single step.

In yet another preferred embodiment the present invention relates to the use or method of the present invention wherein the hemeprotein is hemoglobin or myoglobin.

The present invention demonstrates myoglobin binding to Ni-NTA resin which is significant because there is currently a need to improve detection of plasma myoglobin. A quick bedside assay that is specific for cardiac myoglobin would broaden the diagnostic value of myoglobin relative to other less reliable plasma markers in patients suffering myocardial injury and ischemia.

In another preferred embodiment the resin is epoxy activated sepharose CL-6B.

In a further preferred embodiment the chelating ligand iminodiacetic acid (IDA) is used in the methods and uses of the present invention instead. A person skilled in the art will also recognize that besides nickel, zinc, cobalt or mangnese may be suitable ions to form a metal chelate resin with an affinity to hemeproteins which make them applicable in the method of the present invention. Different combinations of metal ions and chelate molecules bound to different resins may prove optimum depending on the biological sample and the application used.

In a still further embodiment the invention relates to a solid support having Ni-NTA affixed thereto in arrayed form.

The term "solid support" as used herein is meant to encompass substrates as, for example, microtitre plates, plastic chambers, membranes, films, tubes, filaments, meshes and particles of different size and shape. The solid support of the invention may also be a porous material which may be coated or derived to alter its binding properties with respect to compounds or components contained in a biological sample. Preferably, the solid support of the invention is a polyurethane film, a plasma polymerized film, a gold coated nylon mesh or a BioCore® sensor

chip. Preferably, the surface is coated with carboxymethylated dextran, surfactants or biotinamidocaproyl derivated chelates.

In yet another preferred embodiment the solid support is a macroporous silica based material, a microtitre plate or a plastic chamber. Preferably the microtitre plate is a polystyrene microtitre plate or the plastic chamber is a TestPak® plastic chamber.

In a further preferred embodiment the solid support is a chip.

In a preferred embodiment of the method of the invention said method is an in vitro method.

In another preferred embodiment said method further comprises the step of reinfunding the so treated blood into said patient.

In addition, the present invention relates to a method of molecular imprinting a hemeprotein or hemeprotein-hemeprotein ligand complex on a metal chelate solid support comprising (a) contacting a hemeprotein or hemeprotein-hemeprotein ligand complex with an array of monomer metal chelate complexes fixed to a solid support; (b) cross-linking or polymerizing said monomer metal chelate complexes; or (a') contacting a hemeprotein or hemeprotein-hemeprotein ligand complex with an array of polymerizable metal chelators followed by fixing the hemeprotein or hemeprotein-hemeprotein ligand complex to a solid support; (b') cross-linking or polymerizing said monomer metal chelate complexes together with other monomers/cross-linkers; and (c) removing said hemeprotein or hemeprotein-hemeprotein ligand complex from said solid support; and (d) testing the molecular imprint that is generated by the template of said hemeprotein or hemeprotein-hemeprotein ligand complex in contact with polymerized metal chelate complexes.

The method of the invention is suitable for generating a memory on a chip of metal chelators that come into contact with hemeprotein. The general technology of molecular imprinting has been described in Bruggeman et al., J. Chromatogr 889

(1-2) (2000), 15-24, Ramstrom et al., Curr Opin Chem Biol 3 (6) (1999), 759-64 and Shi et al., Nature 398 (1999), 593-597. The person skilled in the art can easily adapt the general teachings, reaction conditions etc provided in said document for performing the method of the present invention.

In a preferred embodiment said hemeprotein is hemoglobin or myoglobin.

In another preferred embodiment said metal chelator is Ni-NTA.

In an additional preferred embodiment said solid support is a macroporous silica based material, a microtitre plate or a plastic chamber.

Finally, in yet another preferred said solid support is a chip.

Brief Description of Drawings

This disclosure may best be understood in conjunction with the accompanying drawings, incorporated herein by references, which show:

Figure 1: Clearance of hemolysis in mouse serum and hemeprotein solutions by nickel nitrilotriacetic acid agarose resin.

Mouse hemoglobin in serum hemolysis (Fig 1A), horse myoglobin (Fig 1B), human hemoglobin (Fig 1C), rabbit reticulocyte lysate (Fig 1D) and horse cytochrome c (Fig 1E) were allowed to interact at room temperature with 150µl of Ni-NTA agarose superflow resin by hand-mixing 1 minute and gravity packing for 2 minutes in eppendorf tubes (UPPER PANEL). Elution of bound myoglobin and hemoglobins was accomplished by addition of imidazole to 40mM final concentration (Fig 1B-1E, LOWER PANEL). Hemeprotien solutions are of 1mg/ml in phosphate buffered saline pH=7.4 in met-heme form or converted to reduced and oxygen bound form by addition of sodium dithionite (reduced: Red).

Figure 2: Hemeprotein absorbance spectra.

Absorbance spectra of solutions were done in 96 well UV plates with data collection every 10nm using Spectra Max plus (Molecular Devices). Hemeproteins of 1mg/ml (Fig. 2a, 2c) and 0.5mg/ml (Fig. 2b, 2d) and protoporphyrin IX 5µg/ml (Fig. 2e).

Figure 3: Separation of non-hemeprotein and hemoglobin using Nickel nitrilotriacetic acid chelation chromatography.

Reticulocyte lysate 60µl (**A**) and lysate derived from 20µl of human whole blood (**B**) were loaded on to Ni-NTA column in 0.5ml BINDING BUFFER: 50mM Tris, 300mM NaCl, 50mM NaH_2PO_4 10mM imidazole, pH=8.0. Column fractions of 0.75ml volume were collected under gravity flow in BINDING BUFFER and then in ELUTION BUFFER: 50mM Tris, 300mM NaCl, 100mM NaH_2PO_4, 40mM imidazole pH=8.0. Absorption measurements at 280nm, 410nm and 540nm of fractions in loading buffer and after exchange to elution buffer (indicated by arrow). Insert: 15% SDS-PAGE and Commassie blue staining of column fractions. Each lane was loaded with 15µl of 1:2 diluted sample.

Figure 4: SDS-PAGE Coommassie stain of cross-linked hemoglobin eluted from Ni-NTA column.

60µl of human whole blood lysate equivalent to 0.1µ moles hemoglobin was loaded on to Ni-NTA column and washed under gravity flow in 50ml of Tris-buffer as described above. The immobilized hemoglobin was crosslinked by addition of 79µM glutaraldehyde in phosphate buffered saline pH 7.4 (PBS) at 8:1 molar ratio glutaraldehyde: Hb for 10 minutes at room temperature. The reaction was terminated by washing the column in 10ml of PBS containing 80µM lysine. Column fractions were collected after washings in 25ml of PBS containing 20mM imidazole (lanes 2 and 3), successive elutions in 1ml of PBS 80mM imidazole (lanes 4 to 7) and after 10ml of PBS 80mM imidazole (lane 8). m=monomers, di=dimers, tri=trimers, tet=tetramers, oli=oligomers

Figure 5: Purification of 6X-His tagged protein from reticulocyte lysate.

Expression of C-terminal hexa his tagged ^{35}S-methionine labeled proteins in TNT T7 reticulocyte lysate *in vitro* transcription –translation coupled system (Promega). **A.** ^{35}S-CYP3A4 and **B.** soluble ^{35}S-MHC class II –peptide fusion protein. SDS-PAGE autoradiography of 3µl reticulocyte lysate reaction mixture (left panel **A** and B) and protein eluted from Ni-NTA column in buffer containing 500mM imidazole after 5 column washings in buffer containing 40mM imidazole (right panel **A** and B). Absorption spectra of 1:100 diluted reticulocyte lysate reaction mixture and undiluted Ni-NTA eluate of ^{5}S-CYP3A4 $(his)_6$ (bottom, A).

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

Example 1: Removal and recovery of hemoglobin from biological samples by Ni-NTA resin.

Binding of hemeproteins and hemoglobin in mouse serum and rabbit reticulocyte lysates to NiNTA agarose superflow resin was measured by batch absorptions (Fig 1) Fast, robust and efficient removal of hemeproteins is underscored by the observation that interactions with NiNTA lead to rapid clearance of hemolysis and solutions containing either myoglobin or hemoglobins. Using several concentrations of purified proteins or hemoglobin in human peripheral whole blood the NiNTA binding capacity and binding efficiency was determined to be 20-30mg Hb/ml resin and 80-90 percent respectively, independent of the hemoglobin source (Fig 2A-C). No binding of porphyrin IX (Fig 2D) or cytochrome c (Fig 1E, Fig 2E) to NiNTA was observed.

Example 2: Separation of hemoglobin in biological samples by NiNTA chromatography.

Rabbit retioulocyte lysate (Promega) and fresh human peripheral blood from normal healthy donor was used as a source of hemoglobin in the binding and purification experiments. Human whole blood was collected in 8% sodium citrate and immediately lysed by the addition of saponin to 0.6% final concentration,

placed on ice for 15 minutes and centrifuged at 4°C 10 min X 14,000g. Aliquot of 120µl lysate (20µl whole blood) was then transferred to fresh 1.5ml eppendorf tube and loaded on to Ni-NTA column in 500µl of buffer containing 50mM Tris-HCl, 300mM NaCl, 50mM NaH_2PO_4, 10mM imidazole, pH=8.0. Column of 1ml Ni-NTA superflow agarose resin (Qiagen) was packed under gravity flow to yield 0.75ml void volume in buffer containing 50mM Tris, 300mM NaCl, pH=8.0. Operation of the column was under native conditions. The imidazole concentrations used enhance specific hemoglobin binding and do not accept donations by low stringency histidine residues on the surface of plasma proteins and soluble intracellular blood proteins.

The binding of hemoglobin present in lysates of rabbit reticulocyte and of fresh human whole blood to Ni-NTA is highly selective as is evident by the finding that non-bound material was eluted close to the void volume of the column with complete absence of heme absorption spectra (Fig 3, fractions 1-4).The fractions collected during extensive column washings in 10mM imidazole (fractions 5-13, Fig 3A and fractions 6-18, Fig 3B) also showed absence of heme and no absorbance at 260-280nm which are indicative of nucleic acids, protein and other ultraviolet-absorbing material. Throughout the washings the entire bound hemoglobin was confined to the top 1-2 cm of the column in a defined layer and none of the wash fractions collected had absorption at 410nm or 540nm. This finding shows that the resin has a remarkably high binding capacity for hemoglobin and does not undergo leaching.

Rapid elution of hemoglobin was achieved with a buffer containing 40mM imidazole (Fig 3A and 3B, fractions indicated by arrow). The eluted protein was examined by SDS-PAGE Coomassie blue staining (Fig 3A, fractions 17-22, Fig 3B, fractions 21-28) and 14 kD hemoglobin subunit (Hb) of greater than 98% purity was identified. The higher molecular weight bands appearing in later elution fractions (Fig 3A, fractions 19-2 1, Fig 3B, fractions 24-26) were of approximately 28kD and 42kD and likely to represent Hb oligomers that are not dissociated by denaturing electrophoresis conditions. The concentration of protein in these fractions based on OD_{280} values, 0.1 to 1.0 mg/ml (Fig 3A) and 0.5 to 1.6 mg/ml

(Fig 3B), was in agreement with the mean values of Hb molar absoptivities at OD_{410} $\varepsilon = 400,000\ M^{-1}\ cm^{-1}$ and OD_{540} $\varepsilon = 50,000\ M^{-1}\ CM^{-1}$.

The method of the present invention was furthermore utilized to cross-link human hemoglobin while it remains bound to the Ni-NTA column (Fig 4). The results demonstrate that fresh whole blood hemoglobin immobilized on Ni-NTA resin is amenable to chemical modification and can be recovered in subsequent steps. The Ni-NTA provides an inert solid phase support of immobilized fresh whole blood hemoglobin and the said hemeprotein is accessible to the cross linking reagent glutaraldehyde. Contact between said cross-linker and hemoglobin is allowed to proceed for period of time sufficient to achieve desired cross-linked products and the reaction is terminated by washings of the column to remove unreacted reagents and optionally by washings that include a quencher such as lysine. The unreacted hemoglobin and cross-linked hemoglobin immobilized on Ni-NTA is recovered by eluting from the Ni-NTA column in buffer containing 80mM imidazole. The eluted fractions are then evaluated by SDS-PAGE Coomassie staining (Fig 4). In addition to bands corresponding to dimeric, trimeric and tetrameric hemoglobin the fractions from successive elutions in 80mM imidazole yield an oligomeric species greater than 62kD. These products are indicative of polymerized hemoglobin (Fig 4). Glutaraldehyde reacts with lysine residues of hemoglobin to produce blood substitutes of such size and dimension (Chang, 1997 and Doyle et al., 1999).

The examples described can be further optimized with respect to the imidazole concentrations. According to the hemoglobin source and the goals of purification the imidazole elutions can be combined with size exclusion chromatography in order to separate the different size cross-linked products in a single step . Prior art cross-linking reactions require isolation of hemoglobin and suspension of said hemeprotein in an aqueous carrier medium free of non-hemeprotein and pyrogens. The present invention bypasses the laborious need for carrying out chemical modifications of hemoglobin in solution.

Example 3: Purification of hexa his-tagged protein produced in rabbit reticulocyte lysate in vitro transcription-translation coupled system.

In vitro coupled transcription-translation of the full length ^{35}S-methionine human cytochrome (^{35}S P450 3A4His$_6$) was produced from cDNA subcloned into pGEM-4Z as previously described (Lytton et al., 1999). C-terminally truncated and hexa his tagged ^{35}S-methionine-labeled MHC Class II ß chain-peptide fusion protein (^{35}S peplAßhis$_6$) was produced from polymerase chain reaction fragments derived from mouse spleen cDNA subcloned into commercial vectors pGEM-3Z or T7 TOPO TA pCR2.1 (Invitrogen) (loc. cit.). The hexa his tagged and radiolabeled proteins were purified from the reticulate lysate reaction mixture using the Ni-NTA column.

The results in Fig. 5 demonstrate that Ni-NTA Agarose resin has unique and dynamic binding properties; mainly that it 1) binds to a hemeprotein in the presence of 10 to 20mM imidazole and 2) permits the elution said hemeprotein over a range of 20 to 40mM imidazole while retaining the hexa his sequence-Nickel nitrilotriacetic acid interactions. Because proteins differ in their requirements for folding and optimum expression the reticulocyte lysate system is often chosen as a convenient and quick way to test cDNAs and designed gene constructs for their capacity to properly encode and produce the desired protein molecule. The Ni-NTA resin thus adds another dimension to proteomics. It will allow investigators to examine radiolabeled his tagged proteins free of hemeproteins and reticulocyte lysate materials. This feature has advantages for 1) protein-protein interactions 2) immunoprecipitation experiments and 3) functional assays in which the activity of sub microgram amounts of expressed protein might be perturbed by hemoglobin presence.

Example 4: Elution of hemoglobin from Ni-NTA with a buffer comprising saturated nitric oxide (NO)

Hemoglobin (Hb) can also be eluted from Ni-NTA by addition of a buffer comprising nitric oxide (NO). As an alternative to imidazole elution, elution with nitric oxide has the advantage of maintaining Ni-NTA resin intact.

The steps involved were:

1) PREPARATION OF SAMPLES: Human Hb (Sigma), rabbit reticulocyte lysate (Promega), whole human blood lysate all containing hemoglobin at 0.125µg/ml in PBS pH=7.
2) BATCH ADSORPTION of 750µl solution to 200µl of Ni-NTA Sepharose resin in 2ml eppendorf tube (as described above). Approximately 5% hemoglobin was non-bound as determined by OD 410nM, OD 540 nm.
3) WASHING the bound protein in 1.5ml volumes: 3 washes in PBS pH 7, followed by 1 wash in PBS 0.3M NaCl pH 5 and 1 wash in 3M NaCl PBS pH 7, followed by 3 final washes in PBS pH 7.
4) PREPARATION OF NITRIC OXIDE SOLUTION: 50ml of PBS was degassed overnight in argon. An anaerobic solution of saturated nitric oxide (NO) in PBS, 15mM NO, was prepared by bubbling NO from a pressurized canister into a sealed bottle containing 50ml degassed PBS.
5) HEMOGLOBIN ELUTION: The immobilized hemaglobin was eluted from Ni-NTA by addition of NO solution from a sealed bottle to of 100µl of the washed Ni-NTA resin with a Hamilton syringe to final a concentration of approximately 0.3mM NO and a volume of 300µl. Hemoglobin was eluted by hand-mixing of the eppendorf tube for 1 minute at room temperature followed by centrifugation for 10 seconds x 10,000g. The supernatant containing the eluted hemeprotein was transferred to a clean cuvette for measurements of changes in hemoglobin absorption spectra and determination of nitrate and nitrite content.

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Claims

1. A method of removing hemeproteins or hemeprotein-hemeprotein ligand complexes from a biological sample comprising
   (a) contacting said biological sample containing said hemeproteins or hemeprotein-hemeprotein ligand complexes with a Ni-NTA resin;
   (b) eluting components other than hemeproteins or hemeprotein-hemeprotein ligand complexes from said biological sample contacted with said Ni-NTA resin with a buffer containing 0 to 20mM imidazole and/or 0 to 3M NaCl; and optionally;
   (c) eluting hemeproteins or hemeprotein-hemeprotein ligand complexes from said Ni-NTA resin with
      (ca) a buffer comprising imidazole in a concentration higher than 20mM; or
      (cb) with a buffer comprising 0.1 to 1mM nitric oxide.

2. A method of removing hemeproteins from a his-tagged fusion protein produced in a rabbit reticulate lysate in vitro transcription-translation system comprising
   (a) contacting crude reticulate lysate containing said his-tagged fusion protein with a Ni-NTA resin;
   (b) eluting hemeproteins present in said crude reticulate lysate with a buffer comprising 20 to 80mM imidazole; and
   (c) eluting said his-tagged fusion protein with a buffer comprising 250mM to 1M imidazole.

3. The method of claim 2, wherein at least one washing step is included after step (a) and prior to step (b).

4. A method of selectively enriching and/or purifying hemeproteins or hemeprotein-hemeprotein ligand complexes or hemeprotein ligands from a biological sample comprising
   (a) contacting said biological sample containing said hemeproteins or hemeprotein-hemeprotein ligand complexes with a Ni-NTA resin;
   (b) washing said biological sample contacted with said Ni-NTA resin with a buffer comprising imidazole in a concentration equal to or lower than 20mM;
   (c) eluting hemeproteins or hemeprotein-hemeprotein ligand complexes or hemeprotein ligands from said Ni-NTA resin with
      (ca) a buffer comprising imidazole in a concentration greater than 20mM; or
      (cb) with a buffer comprising 0.1 to 1mM saturated nitric oxide; or
      (cc) with a buffer comprising reagents necessary to dissociate hemeprotein-hemeprotein ligand binding interactions, and optionally
   (d) lyophilizing the eluate or crystallizing the hemeproteins or hemeprotein-hemeprotein ligand complexes from the eluate.

5. The method of claim 4, wherein elution is performed in step (ca) with a buffer comprising imidazole in a concentration of 25 to 80mM.

6. The method of claim 3 or 4, wherein at least two washing steps are carried out.

7. The method of any one of claims 3 to 5, wherein said washing steps are effected with a phosphate buffer or a Tris buffer.

8. The method of any one of claims 4 to 6 comprising the further step of lysing whole cells prior to step (a).

9. A method for the removal of extracellular hemoglobin or hemeprotein-hemeprotein ligand complexes from patients affected with thalassemia,

hemochromatosis, hemolytic anemia, or any other disease or condition in which the hemoglobin or hemeprotein-hemeprotein ligand complexes presence leads to injurious effects or patients suffering from hemoglobin-lipopolysaccharide septic shock, hemoglobin-nitric oxide mediated septic shock, hemoglobin-nitric oxide induced imbalances, hemolysis conditions emanating from extra corporal oxygenation or prosthetic devices or exposure to drugs, chemicals, poisons or bacterial toxins comprising conducting peripheral blood from said patient through an extracorporeal hemoglobin or hemeprotein-hemeprotein ligand complexes adsorbent system, wherein a Ni-NTA resin adsorbent is built into an appropriate membrane tubing or sheet material to permit blood flow without lysis of red blood cells.

10. A method for the detection of hemeproteins or hemeprotein-hemeprotein ligand complexes in a sample, the method comprising
    (a) applying the sample to Ni-NTA immobilized on a substrate;
    (b) washing the immobilized Ni-NTA with a buffer comprising 0 to 20mM imidazole thereby eluting components other than hemeproteins or hemeprotein-hemeprotein ligand complexes from the immobilized Ni-NTA;
    (c) eluting hemeproteins or hemeprotein-hemeprotein ligand complexes from the substrate with a buffer comprising imidazole in a concentration higher than 20mM or with a buffer comprising 0.1 to 1mM nitric oxide;
    (d) quantifying the eluted hemeproteins or hemeprotein-hemeprotein ligand complexes.

11. The method of any one of claims 1 and 4 to 9 wherein said biological sample is whole blood lysate, plasma, serum, a tissue extract, a recombinant hemoglobin or hemeprotein-hemeprotein ligand complexes preparation, a blood substitute or any other sample containing hemeproteins or hemeprotein-hemeprotein ligand complexes.

12. The method of claim 8, wherein said patient is a mammal.

13. The method of claim 11, wherein said mammal is human.

14. The method of any one of claims 1 to 12, wherein said buffer is a physiological buffer having a pH value of 5 to 8.

15. The method of claim 13, wherein said pH value is between 7 and 8.

16. The method of any one of claims 1 to 14, wherein said buffer in step (b) comprises 0.1 to 3M NaCl.

17. The method of any one of claims 1 to 15, wherein the Ni-NTA resin is Ni-NTA agarose.

18. The method of any one of claims 2, 3, 5, 6 and 16, wherein said his-tag is a 6X-his-tag.

19. Use of Ni-NTA resin for the removal of hemeproteins from a biological sample from a his-tagged fusion protein produced in a rabbit reticulate lysate in vitro transcription-translation system.

20. Use of Ni-NTA resin for the enrichment of hemeproteins or hemeprotein-hemeprotein ligand complexes from a biological sample.

21. The use of claim 18 or 19, wherein said biological sample is blood.

22. The use of claim 20, wherein said Ni-NTA resin is Ni-NTA agarose.

23. A method for the preparation of a stromal free blood substitute comprising the steps of

a) preparing a whole blood lysate;

b) contacting said whole blood lysate with a Ni-NTA resin;

c) eluting components other than hemoglobin from said biological sample contacted with said Ni-NTA resin with a buffer comprising imidazole in a concentration equal to or lower than 20mM;

d) cross linking the resin-bound hemoglobin with an appropriate reagent in an appropriate buffer;

e) chemical modification of the resin bound hemoglobin;

f) eluting the resin-bound hemoglobin from said Ni-NTA resin with a buffer comprising imidazole in a concentration equal to or higher than 20mM or with a buffer comprising 0.1 to 1mM nitric oxide; and

g) gel filtration in a size exclusion matrix.

24. The method of claim 23, wherein the elution in step (f) is performed in a buffer comprising imidazole in a concentration of about 20 to 200mM thereby separating hemoglobin tetramers from oligomers and uncross-linked monomers.

25. The method of any one of claims 1 to 17 and 22 or 23 or the use of any one of claims 18 to 21, wherein the hemeprotein is hemoglobin or myoglobin.

26. The method of any one of claims 1 to 17, 22 to 24 or the use of anyone of claims 18 to 21, wherein the resin is epoxy activated sepharose CL-6B.

27. A solid support having affixed thereto in arrayed form a Ni-NTA resin.

28. The solid support of claim 26, which is a macroporous silica based material, a microtitre plate or a plastic chamber.

29. The solid support of claim 27 or 28 which is a chip.

30. The method of claim 9 wherein said method is an in vitro method.

31. The method of claim 9 further comprising the step of reinfunding the so treated blood into said patient.

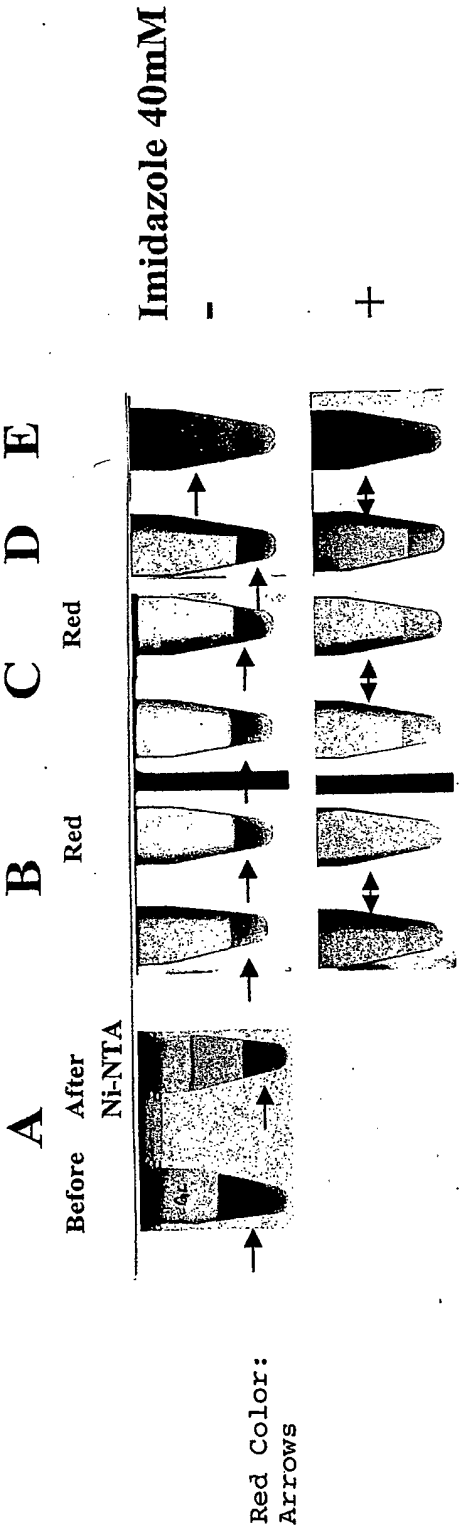
32. A method of molecular imprinting a hemeprotein or hemeprotein-hemeprotein ligand complex on a metal chelate solid support comprising
    (a) contacting a hemeprotein or hemeprotein-hemeprotein ligand complex with an array of monomer metal chelate complexes fixed to a solid support;
    (b) cross-linking or polymerizing said monomer metal chelate complexes; or
    (a') contacting a hemeprotein or hemeprotein-hemeprotein ligand complex with an array of polymerizable metal chelators followed by fixing the hemeprotein or hemeprotein-hemeprotein ligand complex to a solid support;
    (b') cross-linking or polymerizing said monomer metal chelate complexes together with other monomers/cross-linkers; and
    (c) removing said hemeprotein or hemeprotein-hemeprotein ligand complex from said solid support; and
    (d) testing the molecular imprint that is generated by the template of said hemeprotein or hemeprotein-hemeprotein ligand complex in contact with polymerized metal chelate complexes.

33. The method of claim 32 wherein said hemeprotein is hemoglobin or myoglobin.

34. The method of claim 32 or 33 wherein said metal chelator is Ni-NTA.

35. The method of any one of claims 32 to 34 wherein said solid support is a macroporous silica based material, a microtitre plate or a plastic chamber.

36. The method of any one of claims 32 to 34 wherein said solid support is a chip.


A
B
C
D
E
Before
After
Ni-NTA
Red
Red
Imidazole 40mM
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+
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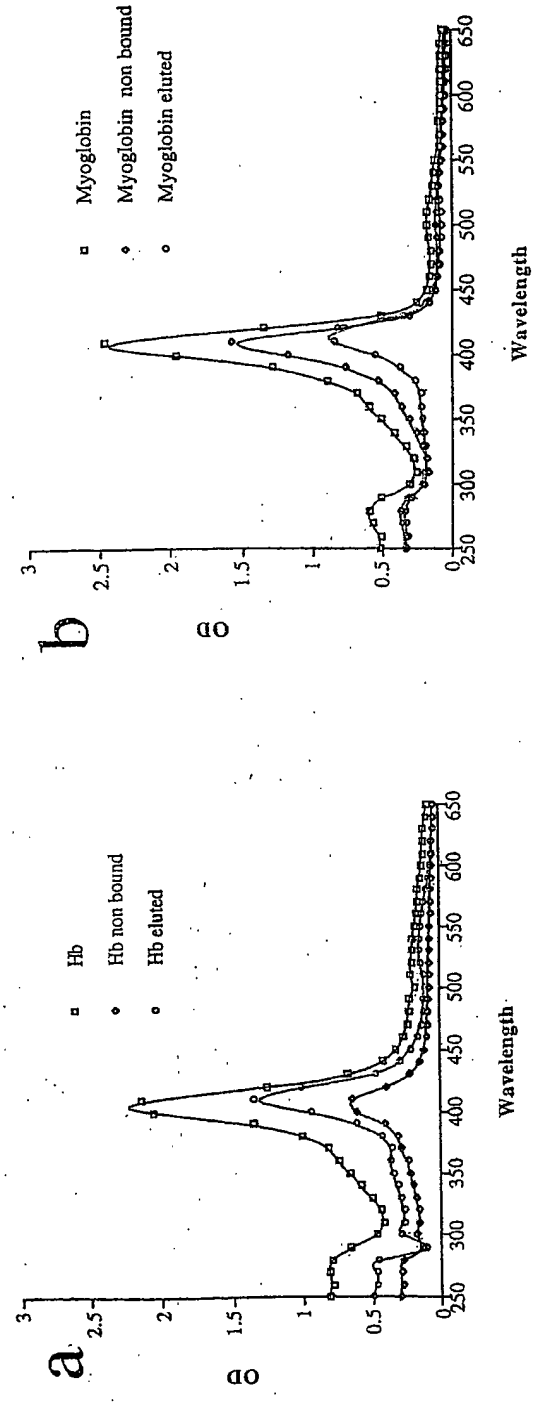
Arrows

Fig. 1


a
b
OD
Wavelength
Hb
Hb non bound
Hb eluted
Myoglobin
Myoglobin non bound
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1.5
2
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3
250
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350
400
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650

Fig. 2

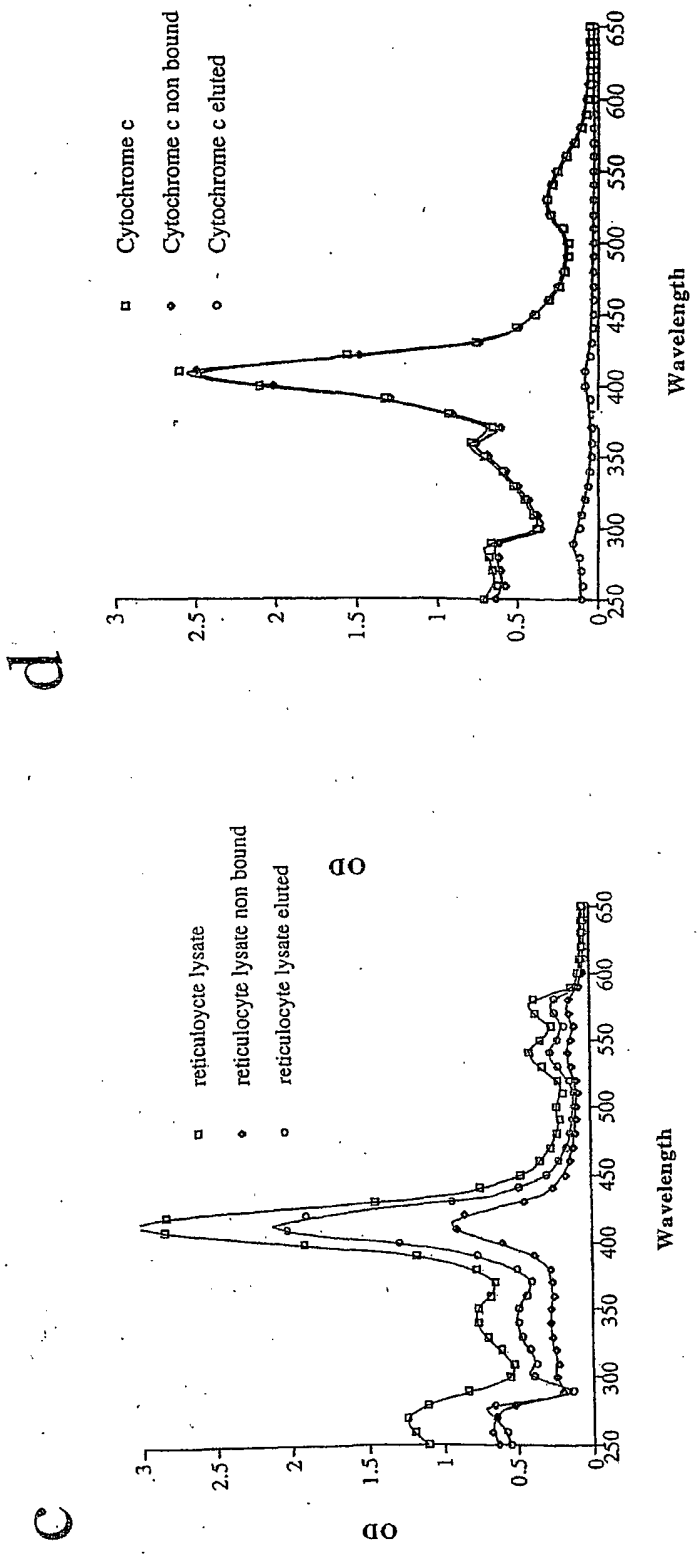

Fig. 2 continued

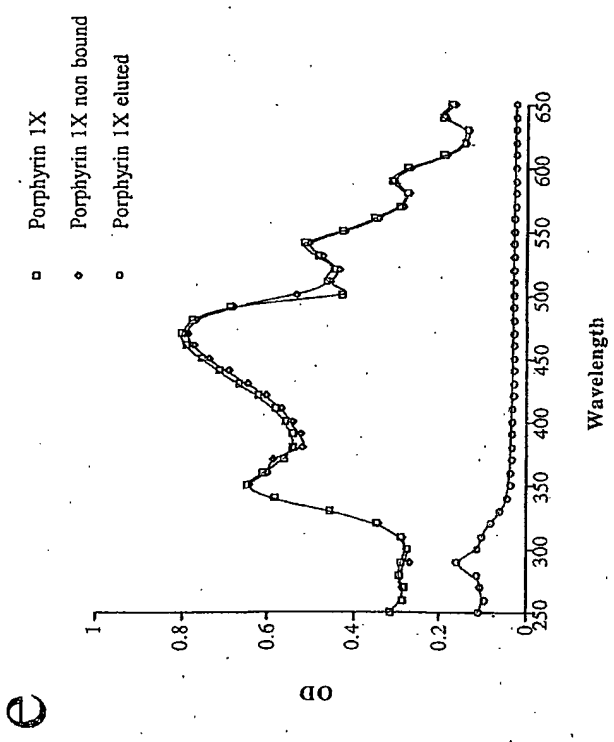

Fig. 2 continued

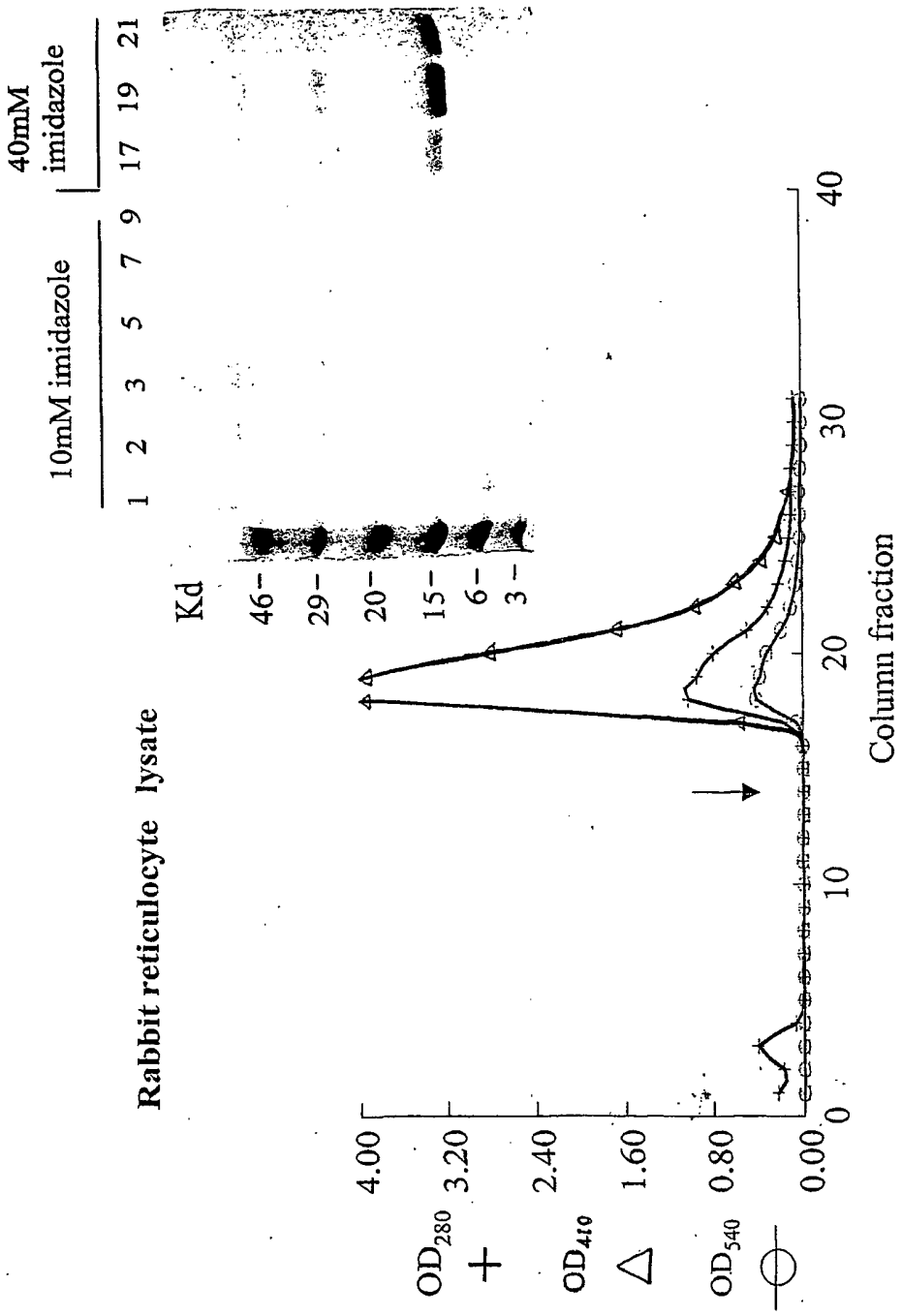
Rabbit reticulocyte lysate
10mM imidazole
40mM imidazole
1 2 3 5 7 9 17 19 21
Kd
46
29
20
15
6
3
OD280 +
OD410 △
OD540 ○
4.00
3.20
2.40
1.60
0.80
0.00
0
10
20
30
40
Column fraction

Fig. 3

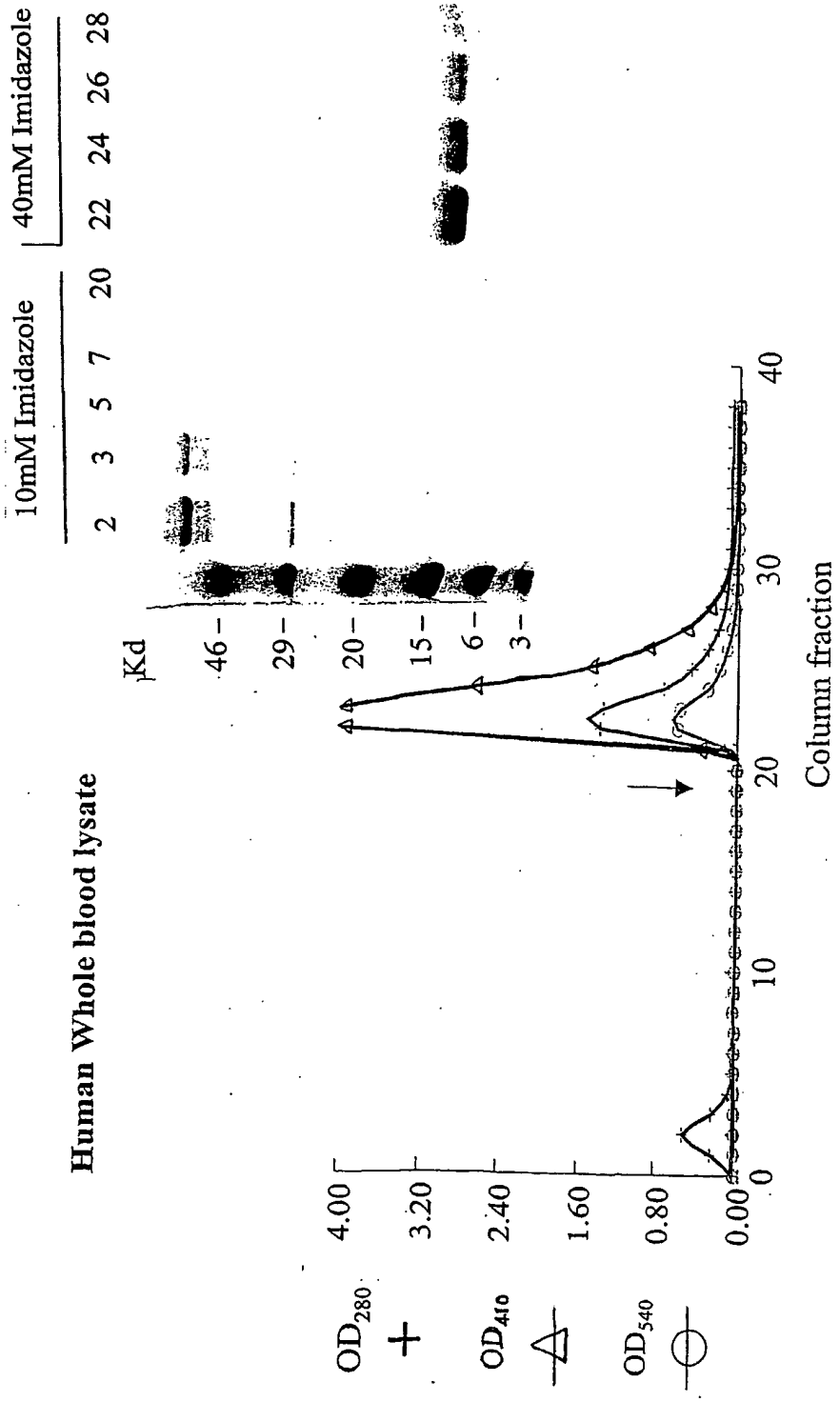

Fig. 3 continued

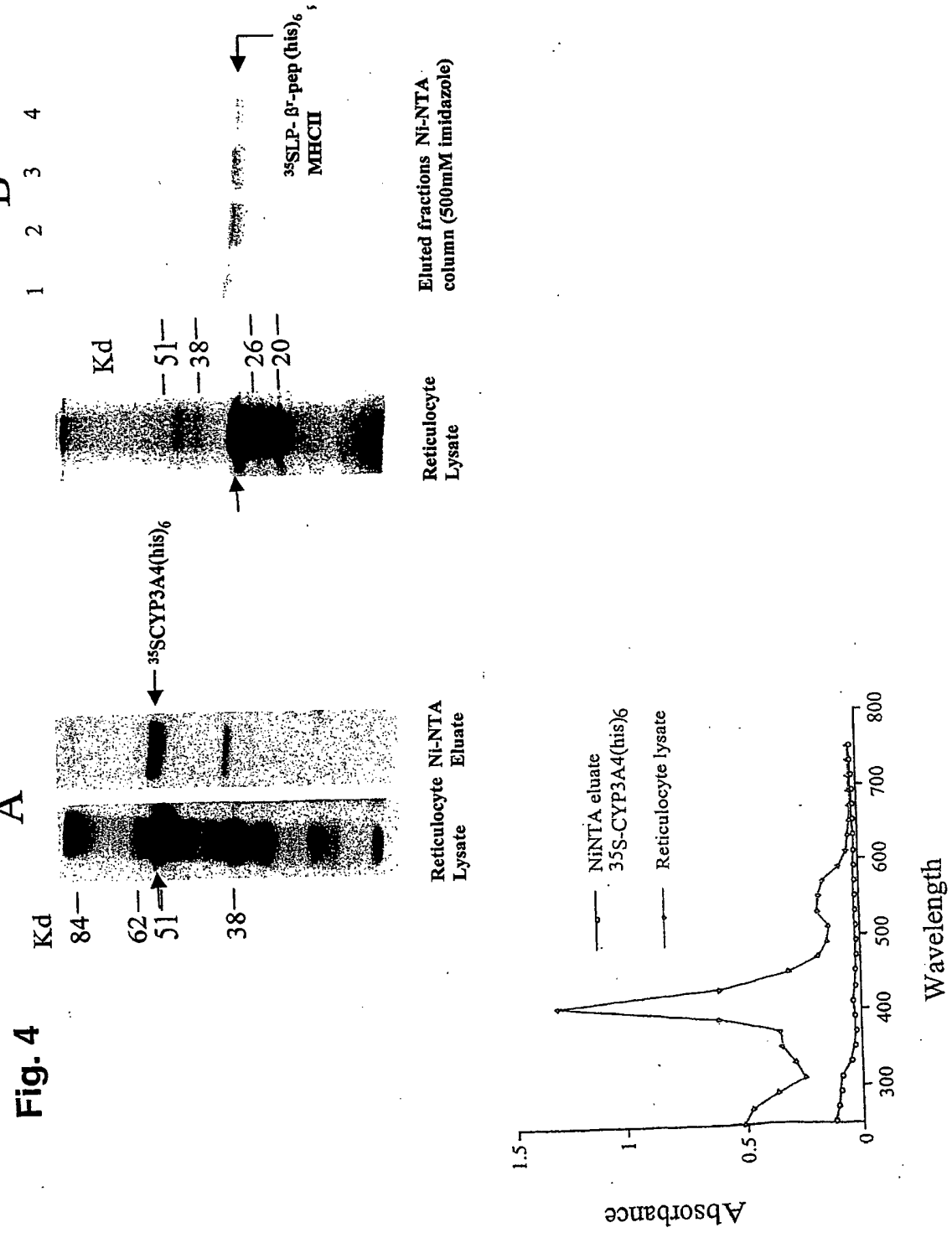
A
Kd
84
62
51
38
^{35}SCYP3A4(his)$_6$
Reticulocyte Lysate
Reticulocyte Ni-NTA Eluate
B
1 2 3 4
Kd
51
38
26
20
^{35}SLP- β^r-pep (his)$_6$
MHCII
Reticulocyte Lysate
Eluted fractions Ni-NTA column (500mM imidazole)
NiNTA eluate 35S-CYP3A4(his)6
Reticulocyte lysate
Absorbance
1.5
1
0.5
0
300
400
500
600
700
800
Wavelength

Fig. 4

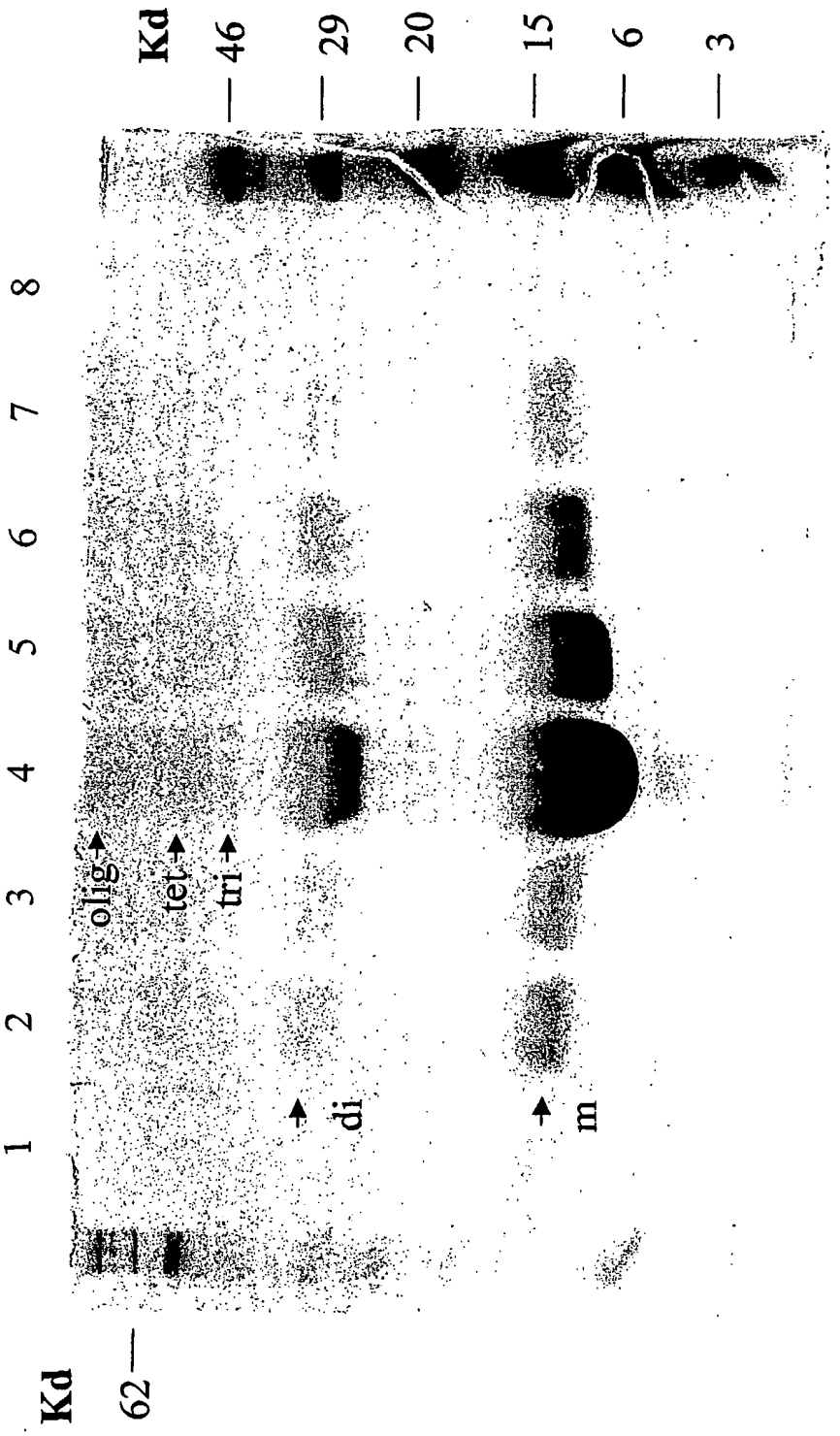

Fig. 5